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(H.C.Bakshi)
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FORM 3A

THE PATENTS ACT, 1970 (39) 655 DEL 99

COMPLETE SPECIFICATION

[See Section 10]

Use of Primaquine Derivative: N^1-(3-Ethylidinotetrahydrofuran-2-one) –N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine as Gametocytocidal agent

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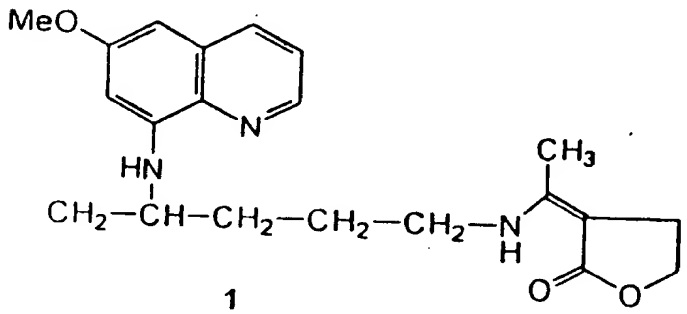
The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed:

Use of Primaquine Derivative : N^1-(3-Ethylidinotetrahydrofuran-2-one)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine as Gametocytocidal Agent.

This invention relates to the use of primaquine derivative N^1-(3-ethylidinotetrahydrofuran-2-one)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine as gametocytocidal agent. More particularly, this invention relates to the use of primaquine derivative N^1-(3-ethylidinotetrahydrofuran-2-one)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine of formula 1 given below useful for controlling the spread of malaria by virtue of its high therapeutic value as gametocytocidal agent. The primaquine derivative of the present invention does not damage either normal or G-6PD deficient erythrocytes to the extent as is observed with the use of primaquine.



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Malaria is one of the most serious protozoal infection in man. According to an estimation made in 90's, about 300 to 500 million people develop clinical infection and one million die of severe infection each year. India is also among the countries to have endemic regions of the disease. It is, therefore, of prime concern and requirement to have therapeutically safe agent

specially which block transmission of malaria through the individuals visiting endemic regions for multiple use. A recent report of resurgence of malaria after a long gap of 40 years from Italy through the transmission, highlights our concern [The Lancet, 350, 717 (1997)]

Malaria is caused by infection with any of the four species of *Plasmodia*. The life cycle of *Plasmodia* is complex and comprises a sexual phase (called sporogony) in the mosquito (a vector) and an asexual division (called schizogony) in human. The life cycle starts after injection of sporozoites by the bite of an infected female anopheline mosquito. Sporozoites then rapidly enter into liver parenchymal cells where they undergo exoerythrocytic schizogony forming primary exoerythrocytic stage or tissue schizonts which mature and release thousands of merozoites in the bloodstream upon the rupture of infected cell. Some of these merozoites enter erythrocytes where they transform into trophozoites and schizonts. The mature schizonts rupture and release merozoites into the circulation which can infect other erythrocytes. This is termed the asexual schizogony (erythrocytic cycle) and it is this periodic release of merozoites which is responsible for characteristic periodicity of the fever in malaria. After several erythrocytic cycles, some erythrocytic forms differentiate into sexual forms called gametocytes. In *P. vivax* and *P. ovale* infections, some of the sporozoites after entering the liver cells are known to remain dormant and form the latent tissue stage called hypnozoites. These hypnozoites upon activation develop secondary tissue schizonts which are responsible for recurrence of malaria called relapsing malaria.

The 8-aminoquinoline antimalarial drugs of which primaquine (PQ) is of exceptional importance, have been demonstrated to possess activity against several life-cycle stages of the parasite. These agents are active against the primary tissue schizonts, thus functioning as causal-prophylactic agents, against the secondry exoerythrocytic forms, thus curing relapsing forms of malaria. The transmission of malaria as discussed earlier is through the injection of sporozoites by the bite of mosquito. These sporozoites develop in the mosquito feeding on an individual carrying mature gametocytes. The male and female gametocytes upon ingestion by a female anopheline mosquito fertilize and transform into zygote and ookinete stages. The ookinetes pierce the epithelium of the midgut where it rounds up into the oocyst. A single oocyst contains as many as 10,000 sporozoites. Primaquine has no sporontocidal activity when provided directly to the insects but has strong gametocytocidal activity and even stops transmission of resistant isolates when mosquitoes are fed on infected blood from primaquine treated animals. Thus primaquine is also a strong transmission blocking agent. However, primaquine even being associated with radical curative and gametocytocidal activities is not in use as a prophylactic agent.

The practical problems associated with use of 8-aminoquinolines are mainly related to their toxicity because of prolonged use in radical treatment required due to the fast metabolism of the drug. Primaquine is known to induce hemolytic lesions in patients suffering from a deficiency in glucose-6-phosphate -dehydrogenase (G-6PD), a genetic condition common among inhabitants of the regions in which malaria is endemic.

Anaemia is a common complication of hemolysis. Primaquine produces metabolites like O-quinone and p-quinonimine functionalities which because of their oxidative nature, oxidize unsaturated fatty acid of erythrocytes causing Red Blood Cell (RBC) lysis. The reduced glutathione (GSH) controls the level of oxidative metabolites and the level of GSH is maintained through NADPH controlled GSSG reduction. NADPH is regulated by G-6PD and hence G-6PD deficient patients are more liable to RBC lysis. As primaquine is the only antimalarial drug which inhibits the development of parasite by interfering at the several stages of parasite life-cycle and therefore an ideal molecule for structural modification to provide a molecule with radical curative and gametocytocidal activities with low toxicity. The study of the fate of primaquine, its metabolites and toxic manifestation in relation with metabolites will therefore, guide the direction of changes in the new molecule. A brief discussion of primaquine metabolism is mentioned here.

Following oral administration of labelled primaquine it was found that 45% of the radioactivity was found in liver tissue, and 22% in the lung, adrenal, spleen, kidney, heart, blood and pancreas while 25% reached into the plasma. Thus primaquine is fairly well absorbed and only a small portion actually reaches the plasma.

Primaquine metabolism occurs at two sites of the molecule : one in the aromatic region at 5- and 6-positions and the other at 8-N-aminoalkyl side chain. The first metabolic pathway leads to the formation of 5-hydroxyprimaquine (5-HPQ, 3), 5-hydroxy-demethyl primaquine (5-HDPQ, 4). The second pathway originally

observed to occur in the micro-organisms, affects the 8-N-aminoalkyl chain and results in the formation of N-acetylprimaquine and desamino carboxylic acid (12). The carboxylic acid derivative (12) is the major metabolite of primaquine in the human plasma.

Strother *et al* identified metabolites from the urine of primaquine treated dogs as 5-hydroxy-6-methoxy-8-(4-amino-1-methylbutylamino)quinoline (3), desmethyl-6-hydroxy-8-(4-amino-1-methylbutylamino)quinoline (9) and 5,6-dihydroxy-8-(4-amino-1-methylbutylamino)quinoline (4) [A. Strother, *et al.*, `Metabolism of 8-aminoquinoline antimalarial agents'. **Bulletin of the World Health Organization**, 59, 413-425 (1981)]. Among N-dealkylated derivatives of primaquine metabolites were identified as 6-methoxy-8-aminoquinoline (10) [J.D. Baty *et al*. The identification of 6-methoxy-8-aminoquinoline as a metabolite of primaquine in Man. **Biomedical Mass spectrometry**, 2, 304-306 (1975)] and 8-(3-carboxy-1-methylpropylamino)-6-methoxy quinoline (12) [J.K. Baker, *et al* `HPLC analysis of the metabolism of primaquine and Identification of a New Mammalian Metabolite' **Journal of Chromatography**, 230, 69-77 (1982)]. A blue color metabolite derived from 5-hydroxy-desmethylprimaquine was identified as tricyclic quinonimine (8) [A.Strother *et al*, `Metabolism of Primaquine by various Animal Species' in **Primaquine: Pharmacokinetics, Metabolism, Toxicity and Activity**, pp 27-48 (1984), John Wiley & Sons].

Primaquine has blood schizontocidal activities whereas its desmethyl derivative has little. Two 5-OH derivatives (3 and 4)

are highly active. The quinolines that lack the side chain at 8-position but have merely amino substituents 10,11 have no significant activity.

In mark contrast is the observation that the dealkylated derivatives 10 and 11 retain their tissue schizontocidal effect. They are 2-3 times more active than primaquine.

The direct sporontocidal activity of PQ and of these putative metabolites is poor against the oocysts development when mosquitoes are fed on treated animals that supply the gametocytes. Primaquine is quite inactive as sporontocide when given directly to the insect, but is very potent gametocytocidal agent.

The 5-hydroxy derivative (4) of desmethyl primaquine shows only a slight gametocytocidal activity. Demethyl primaquine (9) and 5-hydroxy (3) and carboxylic acid (12) metabolites of PQ are all inactive. Of particular interest is the observation that two of the quinolines 10 and 11 with unsubstituted $-NH_2$ group on 8-position are directly sporontocidal. [W. Peters *et al*. 'The activity of primaquine and its possible metabolites against rodent malaria'. **Primaquine : Pharmacokinetics, Metabolism, Toxicity and Activity** pp 93-101 (1984) John Wiley & Sons].

Toxicity of Primaquine and its Metabolites : Primaquine (2) itself appears to have little oxidant activity even when incubated with G-6PD deficient erythrocytes [I.M. Fraser *et al* 'Effects of Drugs and Drug Metabolites on Erythrocytes from Normal and Glucose-6-phosphate Deydrogenase Deficient Individuals'. Annals of New York Academy of Sciences , 151, 777-94 (1968)], whereas 5-hydroxyprimaquine (3) and 5,6-dihydroxy-8-

amin-quinoline (11) cause oxidation of oxyhemoglobin (HbO_2) to methemoglobin (Met Hb) and of reduced glutathione (GSH) [K.A. Fletcher *et al.* 'The Pharmacokinetics and Biochemical Pharmacology of Primaquine in Rhesus Monkeys and Rats' in Primaquine : **Pharmacokinetics, Metabolism, Toxicity and Activity,** pp 49-63 (1984), John Wiley & Sons]. These compounds were also shown to have significant activity with aged, glucose-starved normal erythrocytes. The oxidative metabolite of primaquine is produced by one-electron oxidation of metabolite and HbO_2, and the autoxidation by molecular oxygen.

Both 5-hydroxy-desmethyl primaquine (4) and the blue compound (8) produced significantly more damage to the G-6-PD-deficient erythrocytes than did primaquine (2). However, the blue compound was less active than 5-hydroxy-demethylprimaquine (4) in oxidizing hemoglobin to Met-Hb and almost as active in decreasing the level of GSH in the cells. [A. Strother *et al.* 'Metabolism of Primaquine by various Animal Species' in **Primaquine: Pharmacokinetics, Metabolism, Toxicity and Activity,** pp. 27-48 (1984). John Wiley & Sons].

The carboxylic acid (12) a major metabolite of primaquine circulating in plasma has not shown any antimalarial activty. It is uncertain whether it contributes significatnly to the toxicity of primaquine although it does not cause methemoglobin formation *in vitro*. Earlier we reported causal prophylactic activity of primaquine derivative namely N^1-(3-acetyl-4,5-dihyro-2-furanyl)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine at 3.16 mg/kgX3 doses against sporozoite induced P.cynomolgi B. infection in monkeys. The derivative also exerts anti relapse (radical

curative) activity at 1 mg/kg X 7 days. (G.P. Dutta, S.K. Puri, V.C. Pandey, M. Seth, A.P. Bhaduri, S.K. Chatterjee, O.P. Asthana and K.C> Gupta, Tropical Diseases, 286 (1998); G.P. Dutta, S.K. Puri, A.P. Bhaduri and M. Seth, Am. J.. Trop. Med. Hyg. 41, 635 (1989). In the derivative, primaquine is substituted at primary amino functionality.

Thus from above survey it is obvious that primaquine possesses antimalarial activities such as blood schizontocidal, tissue schizontocidal and gametocytocidal which are also exhibited by its metabolites. Primaquine is even more active than its metabolites. The carboxylic acid **12** though a major metabolite, is nonfunctional. The metabolites of primaquine are also responsible for its toxicity. The tricyclic metabolite **8** is active but less toxic which therefore, suggest the significance of intact side chain. Therefore, if primaquine molecule is manipulated through the side chain possibly toxicity could be modulated. Secondly primaquine is absorbed and metabolized very fast and as a consequence oxidative burst accrued very fast. Therefore its controlled delivery may result in less toxicity. This led us to prepare primaquine prodrug of less toxic profile. Primaquine is of a basic nature with a free amino functionality which is a point of metabolism for inactive metabolite. We derivatised this amino functionality to enaminone and evaluated its efficacy for gametocytocidal action and methemoglobin toxicity. Enaminones are a functional group for controlled delivery of amino drugs. An enaminone derivative of a physiologically active amines may well show improved transport across biological membranes and allow a high concentration of the

amine to be released close to the site of action. This functional group provides resistance towards hydrolytic cleavage at acidic pH as compared to the plain enamine. We prepared enaminone derivative 1 of primaquine on two accounts. Firstly it should have slow metabolic degradation through side chain and secondly compound of enhanced lipophilic character should penetrate better in the tissue, especially in the liver where hypnozoites reside. We therefore, embarked upon to prepare enaminone derivative 1 and the results of its gametocytocidal activity and safety profiles are mentioned here. As already mentioned at outset of the manuscript the search of a safe gametocytocidal agent is needed for two reasons firstly to block the reoccurance of malaria in non-endemic regions where malaria has already been erradicated through vector control methods by individuals visiting endemic regions and secondly it blocks spread of even resistant strains also.

1. The main object of the invention is to provide a new primaquine derivative with the enaminone functionality having gametocytocidal activity and low toxicity for use as transmission blocker.

2. Another object of the invention is to provide a new primaquine derivative for facilitating controlled delivery of amino drugs.

3. Another object of the invention is to provide a primaquine derivative having slow metabolic degradation through the side chain modification.

4. Another object of the invention is to provide a primaquine derivative with enaminone functional group providing resistance

towards hydrolytic cleavage at acidic pH as compared to the plain enamine.

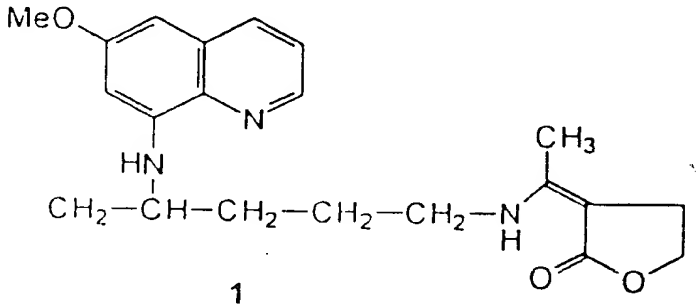
5. Another object of the invention is to provide a primaquine derivative of enhanced lipophilic character to facilitate better penetration in the tissue especially in the liver where hypnozoites reside.

6. Another object of the invention is to provide a primaquine derivative having high therapeutic index ratio in terms of methaemoglobimia formation.

7. Another object of the invention is to provide for a primaquine derivative which causes the oxidation of glutathione (GSH) to the lesser extent.

8. Another object of the invention is to provide a process for the preparation of the novel derivative of formula 1.

Accordingly, the invention provides a new use of primaquine derivative of the formula 1 shown below with the enaminone functionality having gametocytocidal activity and low toxicity for use as transmission blocker.



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In an embodiment, the invention provides the use of primaquine derivative for facilitating controlled delivery of amino drugs.

In yet another embodiment, the invention further provides

the use of primaquine derivative having slow metabolic degradation through the side chain modification.

In a further embodiment, the invention provides the use of primaquine derivative with enaminone functional group providing resistance towards hydrolytic cleavage at acidic pH as compared to the plain enamine.

In yet another embodiment the invention provides the use of primaquine derivative having enhanced lipophilic character to facilitate better penetration in the tissue especially in the liver where hypnozoites reside.

Another embodiment further provides the use of primaquine derivative having high therapeutic index ratio in terms of methaemoglobimia formation.

In another embodiment, use of primaquine derivative causes the oxidation of glutathione (GSH) to the lesser extent.

The primaquine derivative used in the present invention has been prepared by known process which comprises synthesis of enaminone : N^1-(3-ethylidinotetrahydrofuran-2-one)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine. Reaction of 8-(4-amino-1-methyl-butyl amino)-6-methoxy quinoline (Primaquine) with 3-acetyl-γ-butyrolactone in presence of a base in catalytic amount provide the required product.

The following example illustrates the details of the process of this invention.

N^1-(3-Ethylidino tetrahydrofuran-2-one)-N^4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine

A mixture of primaquine base (0.97g, 3.7 mmole) freshly distilled 3-acetyl-γ-butyrolactone (1.0g, 7.8 mmole) and base like piperidine (2-3 drops) were stirred under magnetic stirrer at room temperature. in an hour or so the reaction mixture solidified. The product titurated in ether and filtered to get the product. It was crystallised from alcoholic solvent like propanol Yield 0.89 g, m.p. 118-120°C.

Gametocytocidal Activity

For the gametocytocidal test, batches of 3-4 day old An. stephensi were allowed to feed on *P. cynomolgi* infected rhesus monkeys at appropriate gametocytaemia level. One hr after the control (pretreatment) feeding, compound 1 was administered to the monkeys at 0.63, 1.25, 1.87, 2.5, 3.75 and 5.0 mg/kg in a single dose by oral route. Post-treatment feeding of batches of healthy mosquitoes was done at different times (5-48 hr). Mosquitoes were maintained at 26 ± 1°C under optimal insectary conditions. The infectivity rate and the oocyst counts were recorded on day 8. Mosquitoes were further maintained in the insectary to record the formation of sporozoites and the absence of sporozoites in some of the batches was also ensured by inoculation of mosquito homogenates into naive monkeys.

Results The gametocytocidal activity of compound 1 was evaluated in 16 rhesus monkeys and the pre-treatment mosquito infectivity results for these monkeys show that the oocyst number for different batches ranged from 13.77 ±9.51 to 125.77 ±62.89 and the percent infectivity varied between 42.55 to 100% (Table 1). Sequential mosquito feedings on a monkey treated at 0.63 mg/kg

dose showed significant reduction in oocyst number and the percent infectivity at +5h and +24 h post-treatment compared to the corresponding control feedings at -1 hr. Salivary gland dissections of the mosquitoes from these batches on day 15 showed the presence of sporozoites, thus indicating that oocysts completed normal sporogonic development. No oocysts were observed over the midguts from mosquitoes fed at +48 hr after drug administration nor were any sporozoites seen in their salivary glands.

Identical results were obtained in the efficacy tests at 1.25 mg/kg in 2/2 monkeys, at 1.87 mg/kg in 2/2 monkeys and at 2.50 mg/kg in 2/3 monkeys. The mosquito batches fed at 4-5 hr post-treatment showed marked decrease in the oocyst numbers, though these oocysts were able to complete the sporogonic cycle as indicated by the presence of sporozoites in salivary glands on day 15-16. The mosquito batches fed on these monkeys at +24 hr and +48 hr did not develop any oocysts nor were any sporozoites demonstrable in their salivary glands.

The oocyst development was completely blocked in the mosquito batches (fed 4-5 hr as well as 24 hr post-treatment in one of the three monkeys treated at 2.50 mg/kg, 5/5 monkeys treated at 3.75 mg/kg and 3/3 monkeys treated at 5.0 mg/kg dose. Moreover, the salivary gland dissections from these batches carried out between days 14-20 post infective blood meal also did not show any sporozoites. The asexual parasitaemia and gametocytaemia levels for different monkeys are also shown in Table 1. Although the gametocytes were persisting in circulation at +24 hr and +48 hr post-treatment, these gametocytes were not infective for *An. stephensi* as indicated by the absence of

oocysts. Mosquito batches fed on the vehicle control monkey at -1 hr, +24 hr, +48 hr and +72 hr showed consistently high percent infectivity and oocyst number in all the four batches.

Infectivity tests were carried out to ensure that there was no sporozoite development in the mosquito batches found negative for oocysts on day 8 following their feeding on drug treated monkeys. Homogenates of 40-50 mosquitoes each from 11 bactches fed on gametocyte carrying monkeys treated with compound 1 at 1.87, 2.50, 3.75 and 5.00 mg base/kg were inoculated into naive rhesus monkeys. None of these 11 monkeys developed patency upto 60 days of observations, indicating complete absence of any viable sporozoites in these batches (Table 1). Similar inoculations made from three pre-treatment (control) batches and one post-treatment batch (from vehicle control) resulted in the development of patent infection in three monkeys on day 9, 10, 10.

A comparison of the mosquito infectivity in batches fed prior to drug administration and at varying intervals after administration of compound 1 has shown drastic reduction of mosquito infectivity and oocyst development. This effect was found to be dose dependent as complete inhibition with 0.63 mg/kg dose was obtained at +48 hr. with 1.25-2.50 mg/kg at +24 hr and the higher doses of 3.75 and 5.00 mg/kg rendered mature gametocytes non-infective to mosquitoes within 4-5 hrs. This rapid decline of the mosquito infectivity is attributable to the gametocytocidal action of drug. The persisting gametocytes circulating at 24-48 hr post-treatment in compound 1 treated monkeys were non-infective to mosquitoes. Studies with primaquine

Table I : Effect of single dose compound 1 on *P. cynomolgi* gamestocytes as determined by their infectivity to *A. stephensi* mosquitoes

Dose mg/kg at 0 hr	Time of mosquito feeding	Parasitaemia/mm^3		Day 7 oocyst record	
		Asexual	Gametocytes	No.of mosquitoes infected/dissected (%infectivity)	oocyst no per gut (Mean±SD)
0.63	-1 hr	48816	1728	27/30 (90.0)	86.74±39.2
	+5 hr			23/51 (45.1)	10.22± 6.8
	+24hr	30024	1404	15/46 (32.61)	2.93± 2.4
	+48hr	23220	756	0/24 (0)	Nil
1.25	-1 hr	126965	1895	34/38 (89.57)	22.35±11.8
	+5 hr			12/57 (21.05)	2.17± 1.7
	+24hr	103846	1516	0/36 (0)	Nil
	+48hr	15914	109	0/24 (0)	Nil
1.25	-1 hr	23712	1026	20/47 (42.55)	14.40±7.29
	+5 hr			15/70 (21.43)	2.60± 1.7
	+24hr	21204	486	0/30 (0)	Nil
1.87	-1 hr	33602	1166	25/30 (83.33)	28.20±18.9
	+4 hr			6/40 (15.00)	1.17± 0.4
	+24hr	18020	530	0/27 (0)	Nil
	+48hr	7208	212	0/24 (0)	Nil
1.87	-1 hr	61560	1026	23/25 (92.0)	80.69±35.7
	+4 hr			18/31 (58.06)	13.00±12.3
	+24hr	42180	798	0/38 (0)	Nil**
	+48hr	5130	228	0/21 (0)	Nil
2.50	-1 hr	33578	1442	36/46 (78.26)	13.72± 9.5
	+4 hr			20/33 (60.61)	2.90± 2.2
	+24hr	45320	927	0/29 (0)	Nil
	+48hr	18025	206	0/21 (0)	Nil
2.50	-1 hr	135464	4130	26/28 (92.86)	125.77±62.8
	+5 hr			11/30 (36.67)	4.64± 2.8
	+24hr	96642	2478	0/30 (0)	Nil
2.50	-1 hr	38081	2147	29/37 (78.38)	55.79±41.0
	+ 5hr			0/33 (0)	Nil**
	+24hr	31075	1243	0/44 (0)	Nil**

3.75	-1 hr	55728	1296	26/27 (96.30)	22.35±15.8
	+4 hr			0/25 (0)	Nil
	+24hr	55808	540	0/28 (0)	Nil
3.75	-1 hr	45796	1070	15/22 (68.18)	22.00±16.3
	+4 hr			0/21 (0)	Nil
	+24hr	25894	535	0/21 (0)	Nil
3.75	-1 hr	68320	2318	33/40 (82.50)	60.64±35.4
	+5 hr			0/30 (0)	Nil**
	+24hr	26108	366	0/30 (0)	Nil**
3.75	-1 hr	48336	954	22/22 (100.0)	97.09±38.2
	+4 hr			0/41 (0)	Nil**
	+24hr	65084	1696	0/27 (0)	Nil**
3.75	-1 hr	40548	1612	38/49 (77.55)	48.11±34.6
	+5 hr			0/31 (0)	Nil**
	+24hr	42904	620	0/25 (0)	Nil**
5.00	-1 hr	37985	1090	29/31 (93.55)	78.17±70.2
	+5 hr			0/33 (0)	Nil**
	+24hr	23544	436	0/26 (0)	Nil**
5.00	-1 hr	192850	4060	23/28 (82.14)	68.74±50.6
	+5 hr			0/26 (0)	Nil**
	+24hr	156310	2030	0/21 (0)	Nil**
5.00	-1 hr	51150	2310	27/34 (79.41)	31.96±23.8
	+5 hr			0/23 (0)	Nil
	+24hr	39600	770	0/25 (0)	Nil
Vehicle Control					
	-1 hr	41640	1320	23/27 (85.19)	19.14± 7.2
	+24hr	45480	1680	30/30 (100.0)	32.00±17.6
	+48hr	42939	2925	24/24 (100.0)	39.44±23.6
	+72hr	305037	1672	25/28 (89.29)	24.38±12.6

* Patent infection developed in 9-10 days in naive monkeys upon inocu of 10 mosquitoes homogenate on day 15 post infective blood meal.

**No patency developed till day 60 in naive monkeys inoculated with mosquitoes homogenate on day 15 post infective blood meal.

Table II : Gam cytocidal Activity of Primaquine

Dose mg/kg at 0 hr	Time of mosquito feeding	Parasitaemia/mm^3		Day 7 oocyst record	
		Asexual	Gametocytes	No.of mosquitoes infected/dissected (%infectivity)	oocyst no. per gut (Mean±SD)
1.00mg/kg	-1 hr	36166	1428	32/40 (80.0)	17.13±10.0
	+5 hr			32/44 (72.7)	13.69± 7.2
	+24hr	28048	526	0/55 (0)	Nil
	+48hr	15332	234	0/40 (0)	Nil
1.00mg/kg	-1 hr	42394	5152		
	+5 hr			25/34 (72.53)	37.14±16.6
	+24hr	26832	3256	36/46 (78.26)	34.08±14.7
	+48hr	12140	635	3/45 (6.67)	2.17± 1.7
3.16mg/kg	-1 hr	29680	1230	0/40 (0)	Nil
	+5 hr				
	+24hr	23112	749	37/51 (72.55)	57.59±31.0
				0/53 (0)	Nil
3.16mg/kg	-1 hr	16824	1026	0/33 (0)	Nil
	+5 hr				
	+24hr	21204	670	20/47 (42.55)	24.4 ± 7.2
				15/46 (32.61)	2.6±1.76
				0/43 (0)	Nil

Table III : Effect of Compound 1 on developing oocysts of *P. cynomolgi* *An. stephensi* mosquitoes

Age of Infection in mosquitoes	Mosquito feeding on drug treated*/ control monkey	Day 8 oocyst record	
		No. of mosquitoes infected/dissected (%infectivity)	oocyst number gut (Mean±SD)
24 hr	10mg/kg	17/20 (85.00)	144.47±60.35
	Control	15/18 (83.33)	133.33±62.30
	50mg/kg	23/27 (85.19)	67.00±43.58
	Control	29/36 (80.56)	66.00±43.48
48 hr	10mg/kg	20/20 (100.0)	133.20±96.22
	Control	19/21 (90.48)	124.05±65.85
	50mg/kg	26/33 (78.79)	46.15±36.70
	Control	28/34 (82.35)	42.57±35.27
72 hr	10mg/kg	22/25 (88.00)	20.36±17.81
	Control	23/28 (82.14)	26.83±19.00
	50mg/kg	25/29 (86.21)	27.16±20.60
	Control	22/32 (68.75)	26.59±22.05
96 hr	50mg/kg	18/26 (69.23)	40.33±27.38
	Control	19/25 (76.00)	47.42±28.46

* Mosquitoes with 24-96 hr old oocysts were allowed to engorge blood from naive monkey administered compound 1at -7 hr of the mosquito feeding

** Patent infection developed on days 9-10 in naive monkeys upon inoculation of 10 mosquitoes' homogenates.

have shown that 3.16 mg/kg dose produced complete gametocytocidal action at +24 h while at 1.00 mg/kg, nearly 98% loss of infectivity was observed (Table II). The completion of sporogonic cycle in 24-96 hr old oocysts exposed to the action of compound 1 at 10-50 mg/kg dose indicates absence of sporontocidal/oocysticidal action of the drug (Table III).

Methaemoglobin Toxicity Studies

Comparison of Primaquine and Compound 1 in Relation to Their Effect on Methaemoglobin

Beagle dogs have been used for obtaining data on the methaemoglobin formation following treatment with compound 1 or primaquine.

Colony bred beagle dogs were maintained in the Kennel House of the Institute and fed with standard diet. Fourteen dogs were divided into five experimental groups as detailed below:

Group I : Three dogs

Primaquine @ 1.0 mg/kg(base) x 7 days

Group II : Three dogs

Primaquine @ 3.0 mg/kg(base) x 7 days

Group III : Three dogs

Compound 1 @ 1.25 mg/kg(base) x 7 days

Group IV : Three dogs

Compound 1 @ 3.75 mg/kg(base) x 7 days

Primaquine or compound 1 was suspended in 0.3% methyl cellulose solution and administered orally in 10 ml. volume via catheter followed by 5 ml water to flush the catheter. Treatment was administered once daily for seven doses (day 0-6), the day of first dose being day 0. The animals were observed for 20-30

minutes for any vomitting 5 ml blood was collected from beagle dogs on day 0, 3, 7, 13 and 25 using potassium-oxalate crystals as anticoagulant. All the estimations/tests were conducted on the same day of collection of blood. Methaemoglobin was assayed by the method of Evelyn and Malloy (1938, J. Biol. Chem., 126, 655-662). The values are recorded in table V. At primaquine antirelapse curative dose against *P. cynomolgi* in monkeys, (Group I, 1.0 mg/kg), the mean Met-Hb values increased by 3.7 fold on day 7. There was then a gradual decline in Met-Hb values by day 25, but the level was still 2.0 fold the pretreatment level. Primaquine administered at three times the curative dose (Group II, 3.0 mg/kg) showed 10.5 fold increase over the corresponding day 0 value, and the elevated levels again declined after treatment and were 2.5 fold higher than pretreatment values on day 25. Compound 1 at curative dose (Group III : 1.25 mg/kg) only marginally increased the Met-Hb values by 1.7 fold on day 7 and slight increase (2.4 fold) over the pretreatment values was observed on day 25. At the higher dose (Group IV : 3.75 mg/kg) the Met-Hb level on day 7 increased by 3.2 fold and the values declined to 1.8 fold of pretreatment values on day 25. The vehicle control group (group V) showed marginal fluctuation of Met-Hb level within the normal limits.

Thus on day 7 at the curative dose level, Met-Hb formation was 2.7 fold lower with test compound as compared to primaquine. Likewise at three times the therapeutic dose, the Met-Hb formation with the test compound was 3.6 fold lower as compared to primaquine.

Table-IV : Methaemoglobin levels (g%) in Beagle dogs after

treatment with primaquine and compound-1

Group	Treatment	Day 0	Day 3	Day 7	Day 13	Day 25
1.	Primaquine 1.0 mg/kg	0.65±0.03	0.85±0.13	2.39±0.23	1.98±0.34	1.33±0
2.	Primaquine 1.0 mg/kg	0.74±0.07	1.94±0.33	7.81±1.48	5.51±1.03	1.86±0.
3.	Compound 1 1.25mg/kg	0.53±0.11	0.87±0.17	0.89+0.29	1.04±0.07	1.26±0.19
4.	Compound 1 3.75mg/kg	0.66±0.15	1.0 ±0.19	2.14±0.89	1.66±0.52	1.18±0.14
5.	Control 1.0 mg/kg	0.64±0.09	0.16±0.09	0.74±0.01	0.65±0.10	0.83±0.

Day 0 = Start of drug treatment Day 7 = 1 day after last dose of drug
Day 13 = 7 days after last dose of drug Day 25 = 19 days after last do
Day 3 = After 3 doses

Reduced Glutathione (GSH) in Human Erythrocytes

Drug induced haemolysis is a serious complication in persons deficient in G-6-PD enzyme. The presence of reduced glutathione (GSH) in erythrocytes control the level of oxidative metabolites. Therefore, drugs which cause lesser oxidation of GSH level are safe. The level of reduced glutathione in erythrocytes of healthy and G-6-PD deficient individuals were measured after incubation with PQ and compound 1 and results are mentioned in table V & VI. G-6-PD deficiency was detected by the fluorescent spot screening test and confirmed by the enzyme assay method. Heparinised blood samples were collected from each individual and after centrifugation, the packed cells were washed three times with cold saline. One ml aliquots of washed cells were then incubated with different concentrations of the drugs ranging from 1 to 5 ug/ml base of PQ diphosphate and equivalent doses ranging from 1.25 to 62.5 ug/ml of compound 1 in a water bath at 37°C with occasional agitation for 3 hr GSH levels were estimated by the method of Bentler et al. [Improved Method for the Determination of Blood Glutathione, J. Lab. Clin. Med., 61, 882-888 (1963)]

Results

Mean erythrocyte GSH levels in the controls (without drug) were significantly lower in the G-6-PD-deficient individuals (29.5±1.86 mg%) as compared to normals (49.91 ± 4.49 mg%).

Normal erythrocytes exposed to different doses of PQ showed a fall in GSH levels, which reached statistical signficance at concentration 10 µg/ml, whereas the same incubated with compound 1 showed significant decrease in GSH levels only at concentration

31.25 µg/ml (Table V).

At concentration of 25 µg/ml and 50 µg/ml of PQ and equivalent doses of compound 1 in G-6-PD deficient erythrocytes, the decrease in GSH level was statistically significant ($P<0.001$) in cow when GSH level compared to GSH levels in the controls. However, the decrese in PQ treated erythrocytes was much pronounce as compared to compound I treated group, this showing the higher safety margin of the new compound.

Percentage decrease in GSH levels was more pronounced in normal and G-6-PD-deficient erythrocytes treated with PQ as compared to compound 1. Statistically significant decreases were observed at concentrations of 25 µg/ml and 50 µg/ml of PQ as compared to the equivalent doses of test compound in both normal and G-6-PD deficient erythrocytes (Table V and VI).

Table V : GSH levels in normal erythrocytes with different doses of primaquine and equivalent doses of compound-1

Primaquine		Compound-1	
Dose (µg/ml)	GSH (mg%) Mean ±SE	Dose (µg/ml)	GSH (mg%) Mean ±SE
Control (No drug)	49.91 ± 4.49	Control (no drug)	49.91 ± 4.49
1.00	43.50 ± 5.70	1.25	44.08 ± 5.80
5.00	39.00 ± 6.16	6.25	42.50 ± 5.85
10.00	29.67 ± 6.49	12.50	38.25 ± 5.68
25.00	19.42 ± 2.83	31.25	31.00 ± 5.15*
50.00	10.37 ± 1.57	62.50	32.75 ± 5.39**

* Comparison of equivalent doses of compound-1 with primaquine
* $P<0.05$** $P<0.01$

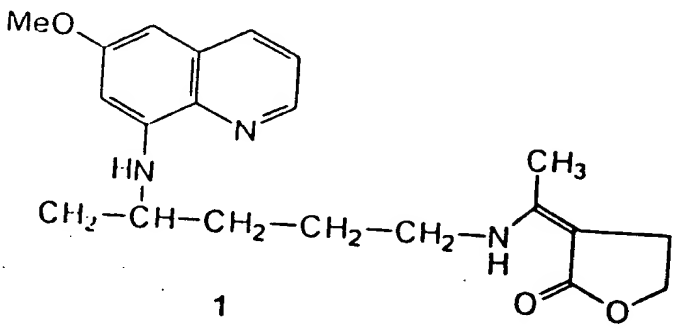
Table VI : GSH levels in G-6-PD deficient erythrocytes with different doses of primaquine and equivalent doses of compound-

Primaquine		Compound 1	
Dose (µg/ml)	GSH (mg%) Mean ±SE	Dose (µg/ml)	GSH (mg%) Mean ±SE
Control (No drug)	29.50 ± 1.86	Control (no drug)	29.50 ± 1.86
1.0	25.75 ± 2.17	1.25	26.04 ± 2.20
5.0	19.17 ± 1.50	6.25	23.42 ± 1.66
10.00	14.83 ± 1.89	12.50	20.00 ± 1.73
25.00	10.50 ± 1.52	31.25	17.17 ± 1.81*
50.00	9.00 ± 1.94	62.50	16.62 ± 1.84**

* P<0.05. Comparison of compound 1 with primaquine

WE CLAIM

1. Use of primaquine derivative of the formula 1 shown below with the enaminone functionality having gametocytocidal activity and low toxicity for use as transmission blocker.



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2. Use of primaquine derivative as claimed in claim 1 for facilitating controlled delivery of amino drugs.

3. Use of primaquine derivative as claimed in claim 1 having slow metabolic degradation through the side chain modification.

4. Use of primaquine derivative of formula 1 with enaminone functional group providing resistance towards hydrolytic cleavage at acidic pH as compared to the plain enamine.

5. Use of primaquine derivative as claimed in claim 1 having enhanced lipophilic character to facilitate better penetration in the tissue especially in the liver where hypnozoites reside.

6. Use of primaquine derivative as claimed in claim 1 having high therapeutic index ratio in terms of methaemoglobimia formation.

7. Use of primaquine derivative as claimed in claim 1 which causes the oxidation of glutathione (GSH) to the lesser extent.

Dated this 29th day of April, 1999.

Indra Dwivedy

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Scientist
IPMD, CSIR
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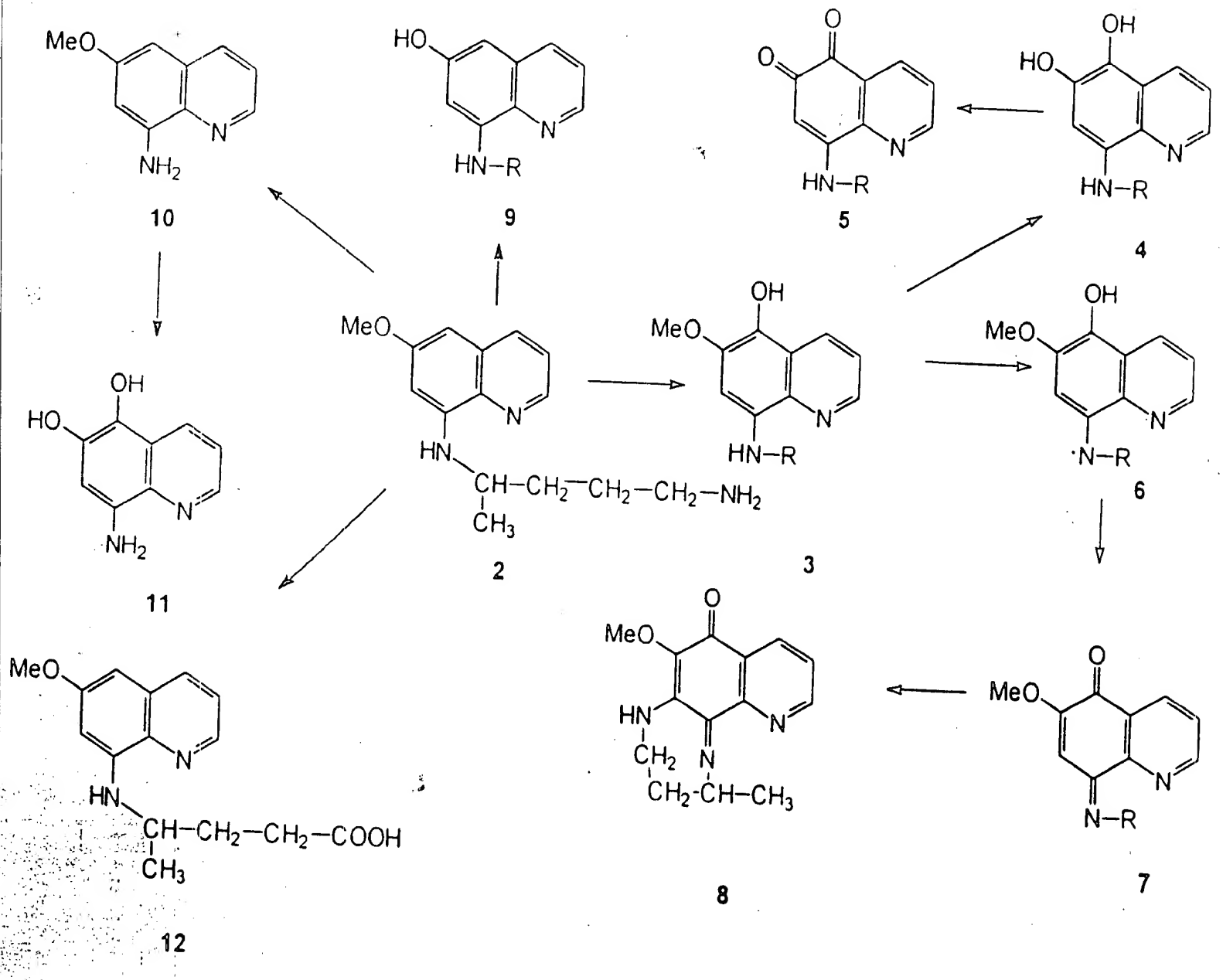

APPLICANTS

Fig. 1 : Primaquine and its putative metabolites

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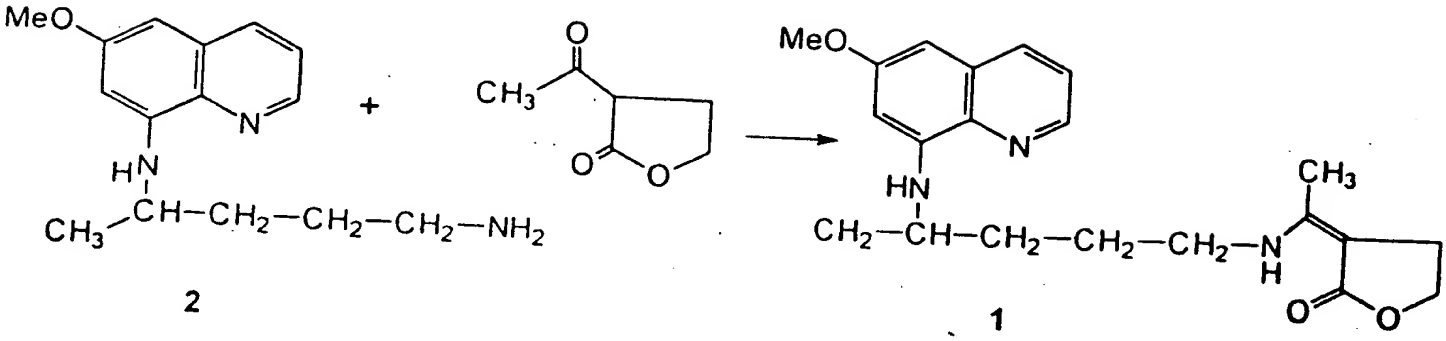

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